Supplementary information

A fluorescent nanosensor for apoptotic cells

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Experimental

All amino acids and peptide-synthesis reagents were purchased from Nova Biochem (EMD Biosciences, Inc., San Diego, CA) and were used without further purification. The dyes, Hoechst 33342, 5-carboxytetramethylrhodamine (5-TAMRA), and 5-carboxytetramethylrhodamine succinimidyl ester (5-TAMRA-SE), were purchased from Molecular Probes (Invitrogen, Carlsbad, CA) N-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was purchased from Molecular Biosciences (Boulder, CO). BioGel P-10 was purchased from BioRad (Hercules, CA). AnxV, FITC-conjugated AnxV (AnxV-FITC), propidium iodide and 7-aminoactinomycin D (7-AAD) were purchased from Sigma-Aldrich (St. Louis, MO). 3-sn-phosphatidylcholine (PC) from egg yolk and 3-sn-phosphadityl-L-serine (PS) from bovine brain were purchased as chloroform solutions (Sigma) and stored at –20 °C. Staurosporine was purchased as a 1 mM solution in DMSO and was stored at –20 °C until needed. Zn(II) complexes were prepared with Zn(NO$_3$)$_2$·6H$_2$O in 50 mM Hepes buffer pH 7.1. Jurkat T cells (clone E6-1) were purchased from ATTC (Manassas, VA) and grown in RPMI 1640 medium enriched with 10% FBS. Cells were grown until their density reached 10$^6$ cells/ml.

Mass spectrometry data were obtained with MALDI-TOF. UV-\textit{vis} spectra were collected on a Varian Cary 50 bio spectrophotometer. Fluorescence measurements were recorded on a Tecan GENios Pro plate reader. Microscopy data were collected on a Zeiss Axiovert 100TV. Confocal microscopy was performed on a Zeiss LSM 5 Pascal Laser.
Scanning Confocal Microscope. Flow cytometry data were collected on a BD FACSCalibur.

**Synthesis of the peptides**

The peptides were synthesized on an automatic peptide synthesizer (Applied Biosystems 433A) in 0.25 mmol scale, using Rink Amide resin with N-α-Fmoc-protected amino acids and standard HBTU coupling chemistry. Diaminopropionic acid (Dpr) with the side chain amino group protected with iv-Dde was chosen. Boc-Gly was selected as last amino acid at the N-terminus. After completion of the synthesis, while the peptides were still attached to the resin, iv-Dde was removed with 2% hydrazine in DMF at r.t. (2 x 15min). The presence of free amino groups was confirmed by the Kaiser test. The amino groups were then derivatized with excess 2-pyridinecarboxaldehyde in dichloroethane by shaking the reaction vigorously shaking for 4 h at r.t.. Excess sodium triacetoxyborohydride was then added to the reaction mixture and the shaking continued for another 4 h. To ensure reaction completion, the resin was subjected to reductive amination twice. The peptides were cleaved from the resin in TFA/thioanisole/ethanedithiol/anisole 90/5/3/2 and recovered by precipitation with methyl t-butyl ether. Peptide purifications were performed on a preparative C$_{18}$ reverse-phase HPLC in water with linear gradients of acetonitrile, in the presence of 0.1% TFA. The identity of the DPA–modified peptides was confirmed by MALDI-TOF. \([M+H]^+\) for mDpr$_4$: 1262.4 calcd.; 1261.6 found. For mDpr$_4$Cys: 1422.6 calcd.; 1422.4 found.
Synthesis of T-mDpr₄

The purified peptide mDpr₄ (15 mg) was reacted with excess of 5-TAMRA-SE (5 mg) in DMSO/Et₃N 80/20 v/v at r.t. overnight. The TAMRA-conjugates were isolated and purified by reverse-phase HPLC. The conjugate identity was confirmed by MALDI-TOF. [M+H]⁺: 1674.9 calcd.; 1675.0 found.

Stock solutions of T-mDpr₄ (10⁻⁴ M) complexed with Zn(II) ions were prepared by adding a slight molar excess of Zn(NO₃)₂·6H₂O in Hepes 50 mM pH 7.1 to T-mDpr₄, dissolved in Hepes 50 mM pH 7.4. The reaction mixture was stirred for 1 h and then lyophilized. It was later redissolved at the desired µM concentration.

Synthesis of T-P-mDpr₄Cys

Iron oxide particles (CLIO-NH₂) were prepared as previously described (Josephson at al, Bioconjugate Chem. 1999, 186). The number of amino group per CLIO-NH₂ particle was determined by reacting with large excess of N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) in DMSO. To CLIO-NH₂ (2 mg Fe), SPDP (75 mM) in 0.1 ml 1X PBS, pH 7.4 was added. The mixture was allowed to stand for 3 hrs at room temperature. Reaction byproducts were removed by Sephadex G-25-80 column (10 - 15 ml), equilibrated with 0.02 M Na – citrate. To measure the number of 2-pyridyl disulfide groups attached, 0.1 mL of the above solution was reacted with DTT (20 mM) in 400 µl of PBS for 2 hours. A microconcentrator, 30 kDa cutoff (Amicon, Beverly, MA) was used to separate the product of the reduction, pyridine-2-thione, from iron. The concentration of pyridine-2-thione was determined using an extinction coefficient at 343 nm of 8100 M⁻¹
1 cm⁻¹. On average, 41 SPSP molecules, representing 41 reactive amino groups, were attached to each CLIO particle.

TAMRA-SE (0.5 mg) dissolved in 50 µL of DMF was added to 360 µL of CLIO-NH₂ (3 mg Fe) in 0.1 M sodium bicarbonate pH 8.5. The reaction mixture was shaken in the dark for 2 h at r.t.. Next, 250 µL of 120 mM SPDP in DMSO was added and the reaction mixture was incubated for an additional 2 h. Unreacted TAMRA and SPDP were removed by gel filtration on a BioGel P-10 column. After purification, the number of TAMRA molecules per nanoparticle was determined spectrophotometrically ($\varepsilon_{550} = 95,000$), and by assuming 2064 iron atoms per nanoparticle. The number of SPDP groups per nanoparticle was determined to be 16 after the release of 2-pyridine-thione from the SPDP-activated CLIO with DTT as described previously. Excess of mDpr₄Cys (0.65 µmol) was then added to the remaining SPDP-activated CLIO-NH₂ and the reaction mixture was allowed to shake overnight at r.t.. The number of mDpr₄Cys peptides conjugated to CLIO was determined by measuring the concentration of 2-pyridine-thione released in the reaction mixture. Unreacted mDpr₄Cys was removed by gel filtration on a BioGel P-10 column. The resulting nanoparticle, T-P-mDpr₄Cys, contained an average of 1 TAMRA and 15 mDpr₄Cys peptides per nanoparticle, and was stored at 4 °C until needed.

Stock solutions (500 µg/mL Fe concentration) of T-P-mDpr₄Cys complexed with Zn(II) ions were prepared by incubating T-P-mDpr₄Cys with a slight molar excess of Zn(NO₃)₂·6H₂O in Hepes 50 mM pH 7.1. The reaction mixture was stirred for 1 h at r.t. and then diluted at the desired concentration.
**Titration with phospholipids**

PS in CHCl$_3$ (750 µL) in increasing concentration, from 0 to 200 µM, were added to 1.5 ml vials, followed by 500 µL of 4 µM TAMRA or T-mDpr$_4$ and Zn(II) complexes, in 10mM Hepes buffer. The vials were manually shaken for 30 s and left to rest for 20 min. The fluorescence remaining in the buffer layer was measured with a plate reader ($\lambda_{\text{ex}}$ 550 nm; $\lambda_{\text{em}}$ 580 nm). The data for each compound were normalized to its intrinsic fluorescence in the absence of PS.

**Flow cytometry and microscopy experiments with apoptotic cells**

Jurkat T cells, at $10^6$ cell/ml concentration, were incubated with 3 µM staurosporine for 4.5 h at 37 °C to induce apoptosis. The percentage of apoptotic cells was estimated by staining with AnxV-FITC and PI and found to be greater than 50%. An analogous group of Jurkat T cells was kept as control. After removal of staurosporine, both the untreated and staurosporine-treated cells was incubated with T-P-mDpr$_4$Cys complexed with Zn(II) ions at 50 µg/mL Fe concentration for 15 min at r.t. Both groups of cells were then stained with AnxV-FITC and 7-AAD for FACS studies. For microscopy studies, the cells were stained with Hoechst 33342, AnxV-FITC and PI.

**AnxV competition experiment**

Jurkat T cells (1 mL), at $10^6$ cell/ml, were incubated with 3 µM staurosporine for 4.5 h at 37 °C to induce apoptosis. After removal of staurosporine, the cells were incubated with T-P-mDpr$_4$Cys complexed with Zn(II) ions at 50 µg/ml Fe concentration for 15 min at r.t.. Then, they were resuspended in 500 µL of AnxV binding buffer (Hepes...
10 mM, NaCl 140 mM and CaCl$_2$ 2.5 mM at pH 7.4). Increasing amounts of unlabelled AnxV, from 0.09 to 70 nM, were added. Binding of AnxV to the apoptotic cells was determined by FACS analysis from the decrease of the TAMRA fluorescence. IC$_{50}$ values of AnxV were determined by the ability to displace T-P-mDpr$_4$Cys in the assay above. The data were fitted to sigmoidal dose-response curves, using Prism 4 (GraphPad Software, San Diego, CA).
Figure S1: **Top:** Images of Jurkat T cells (20X magnification) stained with Hoechst (blue), AnxV-FITC (green) and T-P-mDpr\(r\)Cys (red); **Bottom:** Images of Jurkat T cells (20X magnification) treated with 3 \(\mu\)M Staurosporine for 4.5 h and then stained with Hoechst (blue), AnxV-ITC (green) and Propidium Iodide (red).